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Serous Adenocarcinoma of the Uterine Cervix: A Clinicopathological Study of 12 Cases and a Review of the Literature

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Key Words

Clinicopathological characteristics · Serous adenocarcinoma · Uterine cervix

Abstract

Background/Aims: To determine the clinicopathological characteristics and potentially associated outcomes in patients diagnosed with serous adenocarcinoma of the uterine cervix. ***Methods:*** The records of surgically-treated patients with pathological stage pT1b–2b serous adenocarcinoma were reviewed. ***Results:*** Of 12 patients with serous adenocarcinoma who underwent radical hysterectomy, five had pT1b1N0 disease, two pT1b1N1, two pT1b2N0, and three pT2bN1. The 5-year overall survival rate for patients with or without parametrial involvement (pT2b vs. pT1b) was 0 and 89%, respectively. The 3-year recurrence-free survival rate for those with or without parametrial involvement was 33 and 89%, respectively. Four patients suffered recurrence, namely one of those who had pT1b (1/9, 11%) and 3 of those who had pT2b disease (100%). The sites of recurrence of pT2b disease were outside the pelvis in all 3 patients. Of these, 2 (67%) had peritoneal spread and 1 distant node metastasis. ***Conclusion:*** While patients with pathological stage pT1b disease may have a relatively favorable outcome after radical surgery, those with more advanced disease have a poor prognosis because of extra-pelvic recurrence.

Introduction

Serous adenocarcinoma of the uterine cervix (SACC) is a very rare tumor, while this histological subtype is common in the ovary, Fallopian tube, and peritoneum. Probably for this reason, details of the clinicopathological features of SACC are largely unknown [1, 2]. Reports in the literature are on very limited numbers of patients, with the largest to date including 17 patients [3], this being the only report concerning the clinicopathological factors and prognosis of more than 10 cases. Of these 17 patients, the prognosis of 9 was made after hysterectomy. The present retrospective study assessed the clinicopathological features and prognosis of 12 patients with SACC who underwent hysterectomy.

Material and Methods

We reviewed the medical records and pathological specimens obtained from all patients with primary cervical carcinoma treated and diagnosed at the Gynecology Division and Pathology Section of the National Cancer Center Hospital, Tokyo, Japan, between 1985 and 2005. All definitive diagnoses were made based on excised specimens.

This study included patients who had had a lesion fulfilling the histological criteria for serous adenocarcinoma according to the criteria of the World Health Organization International Histological Classification of Tumors and who had undergone a pri-

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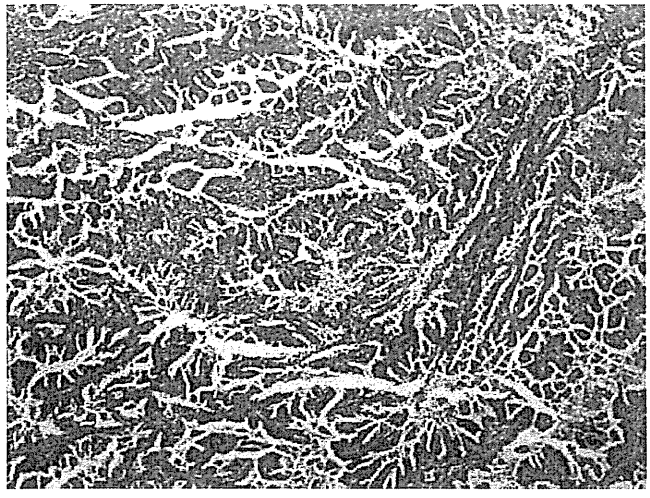

Fig. 1. Papillary serous adenocarcinoma. The tumor shows slender papillae with fibrovascular cores lined by small cells. HE stain, ×40.

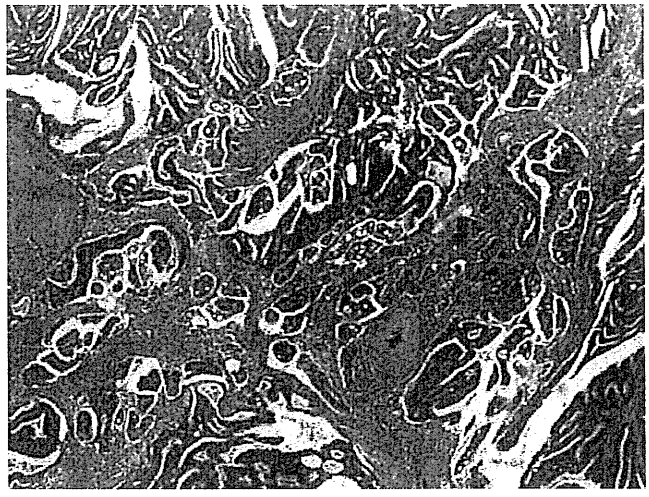

Fig. 2. Papillary serous adenocarcinoma invading cervical stroma, with clusters of tumor cells. HE stain, ×100.

mary hysterectomy. The diagnosis of serous adenocarcinoma was made only when an invasive cervical adenocarcinoma exhibited a prominent papillary structure and/or slit-like glandular spaces, and usually moderate to marked cytologic atypia (fig. 1, 2) without either intra- or extra-cytoplasmic mucin. At least 10% of the tumor area had to be of papillary serous type for inclusion in this study.

For this study, 2 gynecologic pathologists re-examined all surgically removed pathological specimens. Postoperative pathological classification was carried out according to the 2002 revision of the International Union against Cancer (UICC) TNM classification of malignant tumors.

Radical hysterectomy has long been a standard treatment option for the patients with FIGO stage IB–IIB disease in our institute. In patients with pelvic lymph node metastasis or parametrium involvement proven by pathological examination following surgery, adjuvant irradiation to the whole pelvis was administered.

Following primary treatment, asymptomatic patients underwent pelvic examination, Pap smear, ultrasound, and serial determination of tumor markers (CA125, CA19-9 and carcinoembryonic antigen) every 4–6 months. Symptomatic patients underwent the appropriate examination where indicated using chest radiography, computed tomography (CT) and magnetic resonance imaging (MRI).

Follow-up continued until January 2009. Recurrence-free and overall survival curves were calculated using the Kaplan-Meier method and were compared by non-parametric survival analysis (log-rank test). A p value of <0.05 was considered statistically significant. JMP software (version 5.0.1; SAS Institute Inc., Cary, N.C., USA) was used for statistical analysis.

Results

Patient Characteristics

Twelve patients met the study criteria. Their characteristics are summarized in table 1. Their median age was 52 years (range 30–74) and median follow-up time including death was 55 months (range 5–127). No patient was lost to follow-up. Baseline evaluation consisted of a complete gynecologic examination that included PAP smears, colposcopy and biopsy, laboratory studies inclusive of pretreatment CA125, CA19-9 and carcinoembryonic antigen as well as diagnostic imaging (CT, ultrasound, and MRI) at the initial visit. Eleven patients (92%) presented with abnormal genital bleeding as the primary symptom, with no other symptoms. The remaining patient (8%) was asymptomatic and was diagnosed based on abnormal cervical cytology. Nine patients had stage pT1b disease (seven with pT1b1 and two with pT1b2), and three had stage pT2b. All patients underwent radical hysterectomy with bilateral salpingo-oophorectomy and pelvic lymphadenectomy. All tumors were completely removed. Adjuvant radiotherapy or chemotherapy was administered to the 4 patients in whom lymph node metastasis or parametrial invasion was proven by pathological examination of resected specimens. Three of these 4 patients received adjuvant radiotherapy to the whole pelvis, for a total dose of 50 Gy, and 1 patient was treated with chemotherapy (cisplatin, doxorubicin and cyclophosphamide). In this case, the primary care doctor selected not radio-

Table 1. Clinicopathological characteristics of the 12 patients with SACC

Patient No.	Age	Pathological stage	Growth pattern	Grade	Histologic type	Depth of invasion, mm (depth ratio)	Length mm	LVSI	Ovarian metastasis	Adjuvant therapy	Recurrent site	Recurrence months	Status (months)
1	44	pT1b1 N0	Endophytic	2	pure[a]	16 (<3/3)	25	+	–	none	NA	NA	NED (54)
2	46	pT1b1 N0	Exophytic	3	pure	5 (<1/3)	40	–	–	none	NA	NA	NED (106)
3	47	pT1b1 N0	Endophytic	3	mixed[b]	9 (<3/3)	21	–	–	none	NA	NA	NED (65)
4	30	pT1b1 N0	Endophytic	2	mixed	12 (<3/3)	35	+	–	none	NA	NA	NED (127)
5	61	pT1b1 N0	Exophytic	2	mixed	6 (<1/2)	31	+	–	none	NA	NA	NED (62)
6	51	pT1b1 N1	Exophytic	3	pure	10 (<1/3)	27	+	+	chemotherapy	lung	2	DOD (5)
7	53	pT1b1 N1	Exophytic	3	pure	15 (<2/3)	30	+	–	radiotherapy	NA	NA	NED (48)
8	68	pT1b2 N0	Exophytic	2	mixed	40 (<3/3)	55	+	–	none	NA	NA	NED (28)
9	50	pT1b2 N0	Endophytic	2	mixed	20 (<3/3)	40	–	–	none	NA	NA	NED (45)
10	51	pT2b N1	Endophytic	2	pure	21 (<3/3)	23	+	–	radiotherapy	peritoneum	43	DOD (51)
11	74	pT2b N1	Endophytic	3	pure	15 (3/>3)	80	+	+	radiotherapy	peritoneum	35	DOD (40)
12	52	pT2b N1	Endophytic	3	pure	17 (3/>3)	20	–	–	none	PALN	12	DOD (28)

SACC = Serous adenocarcinoma of the uterine cervix; LVSI = lymph-vascular space involvement; NA = not applicable; PALN = para-aortic lymph node; NED = no evidence of disease; DOD = dead of disease. + = positive; – = negative.

[a] Only serous adenocarcinoma was observed; [b] another pathological subtype (endocervical and endometrioid) was observed.

therapy but chemotherapy because he thought that chemotherapy was suitable for cervical serous adenocarcinoma at that time. For 7 patients without lymph node metastasis or parametrial invasion as evaluated histopathologically, no adjuvant therapy was performed. The remaining patient with pT2bN1 disease elected not to receive adjuvant therapy.

Pathological Features

There were large macroscopic tumors (20–80 mm), located in the uterine cervix. Five tumors showed an exophytic pattern and 7 an endophytic one. In 5, another pathological subtype of uterine cervical adenocarcinoma was observed. Three cases had endometrioid adenocarcinoma accounting for 30–60% of the tumor, whereas the other 2 had endocervical-type mucinous adenocarcinoma accounting for 50–70% of the tumor. These 12 cases were classified into 3 cytologic grades according to Zhou's criteria [3]. Six were grade 2, with moderate nuclear pleomorphism, small nucleoli, and moderate amounts of cytoplasm. The other 6 were grade 3, with marked nuclear pleomorphism and prominent nucleoli. All tumors, regardless of grade, had >10 mitotic figures per 10 high-power fields. Psammoma body was present in 1 of the grade 2 cases.

The patients were excluded if they had a history of previous primary serous carcinoma of the ovary, Fallopian tube, endometrium, or peritoneum. There were no serous adenocarcinoma lesions of the Fallopian tube or peritoneum. In 9 cases, tumor extent was confined to the uterine cervix, but 1 case had microscopic ovarian metastasis, and in 1 other, the primary tumor had started to invade the endometrium. Another case had both microscopic ovarian metastasis and myometrial invasion, but the size of the cervical tumor was 80 mm. These ovarian metastatic lesions were extremely small (1 and 6 mm) compared with the cervical mass and were present not in the parenchyma but on the surface of the ovary. In order to distinguish between primary cervical cancer and metastasis from serous ovarian cancer, 2 gynecologic pathologists re-examined all surgically removed pathological specimens.

Survival

Four (33%) patients suffered tumor recurrence after a median of 23 months following initial surgery (range 2–41 months). Two of these patients presented with symptoms of dyspnea caused by pleural effusions, and back problems related to peritoneal recurrence. All 4 of the patients with recurrent tumor died at a median of 9 months after the onset of recurrence despite intensive multimodal therapy (systemic chemotherapy, radiation and surgery).

For all 12 patients, the 5-year overall survival rate was 62% and 3-year recurrence-free survival (RFS) was 74%. The 5-year overall survival rate for patients with or without parametrial involvement (pT2b vs. pT1b) was 0 and 89%, respectively (fig. 3). This was statistically significant ($p = 0.01$). The 3-year RFS rate for patients with or without parametrial involvement was 33 and 89%, respectively, which was also statistically significant ($p = 0.01$).

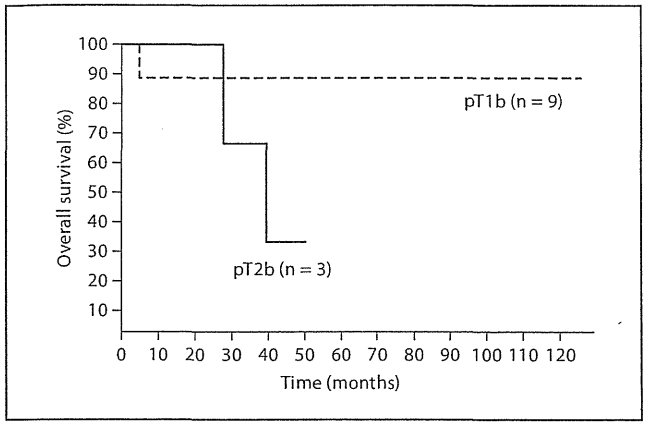

Fig. 3. Overall survival for 12 patients with SACC stratified by clinical stage.

Three-year RFS for patients with lymph node metastasis was 40 compared to 100% for those without. There was a significant difference in RFS between these 2 groups ($p = 0.005$).

The average number of resected lymph nodes was 28 (range 18–48). Pelvic lymph node metastasis was found in the surgically resected specimens from 1 (12.5 %) of the 8 patients with no recurrence, but in all 4 of those whose cancer recurred. All patients with 2 or more positive pelvic lymph node metastases suffered recurrence. Neither the one patient with only 1 positive pelvic lymph node metastasis nor any of the patients without metastasis suffered recurrence.

Recurrence Site

The initial sites of recurrence were located outside the pelvis in all 4 patients. The most frequent site of distant metastasis was the peritoneum (2/4, 50%), followed by the lung (1/4, 25%) and para-aortic lymph nodes (1/4, 25%).

Discussion

SACC is a very rare tumor. No large-scale multicenter study has been performed and the optimal primary therapeutic approach to SACC has not been determined. Several cases have been reported, but only very limited clinical data on SACC are available. The existing reports mostly provided information on the morphologic features and the behavior of this entity, but no accurate staging and assessment of lymph node metastasis based on reviewing surgical specimens for half the patients. Using 'serous adenocarcinoma' and 'uterine cervix' as key words, we conducted a Medline search of articles on SACC published in English from 1984 to 2008, and extracted papers describing accurate surgical staging, sites of recurrence and outcomes.

The literature provides information on a total of 25 patients including those in the present study. Twenty-one (84%) out of these 25 patients underwent radical hysterectomy [3–8]. The clinical characteristics of all 25 patients are summarized in table 2. The pathological stages were one case of pT1a, nineteen of pT1b, two of pT2a and three of pT2b. One patient with pT2a disease was categorized as advanced stage because of the presence of para-aortic lymph node metastasis. Recurrence occurred in 9 patients (three with pT1b disease, two with pT2a, three with pT2b, and one whose status was not mentioned), of whom 7 died of the disease. The recurrence rate in early (pT1 and pT2aM0) and advanced stage (pT2b and pT2aM1) was 23.8% (5/21) and 100% (4/4), respectively. Advanced stage was associated with poor prognosis.

Fregnani et al. [9] reported that the recurrence rate of patients with early-stage adenocarcinoma (FIGO stages IB and IIA) was 11.4% (4/35) and the 5-year RFS rate was 87.9%. Grisaru et al. [10] reported that the 5-year RFS rate of FIGO stage IA–IB patients with common-type adenocarcinoma (mucinous or endometrioid adenocarcinoma) was 90%. In both studies, all patients had undergone radical hysterectomy with or without adjuvant therapy. Kasamatsu et al. [11] reported that the recurrence rate for early-stage (pT1b–2a) patients with common-type adenocarcinoma (mucinous or endometrioid adenocarcinoma) was 16.7% (17/102) and their 3-year RFS rate was 91% and 100% for pT1b and pT1a, respectively. Although the recurrence rate in early-stage patients in the present review of the literature seems high (23.8%), 2 patients whose cancer recurred had not been treated with radical hysterectomy, but only with simple hysterectomy.

A radical hysterectomy with or without adjuvant therapy for early-stage SACC appeared to be associated with a favorable prognosis almost identical with common-type cervical adenocarcinoma. We suggest that the biological behavior of early-stage SACC is similar to common-type adenocarcinoma. On the other hand, all patients with advanced-stage SACC suffered recurrence, despite radical hysterectomy. In our institute, Kasamatsu et al. [11] reported that the 3-year RFS rate for patients with pT2b common-type adenocarcinoma (mucinous or endometrioid adenocarcinoma) who underwent radical

Table 2. Outcome and patterns of recurrence in 25 patients with SACC who underwent surgery: survey of the literature

	Postsurgical stage (n)	Age (mean)	Surgery (n)	Adjuvant therapy (n)	Lymph node metastasis (n)	Recurrence site (n)	Status (n)
Gilks et al. 1992 [4]	pT1b[a]	32	RH	Radiotherapy	Negative	None	NED
Shintaku et al. 1993 [5]	pT2a (1)	66	SH	Radiotherapy	Positive	Peritoneum	DOD
Rose et al. 1993 [6]	pT1a (1)	30	RH	None	Negative	None	NED
Zhou et al. 1998 [3]	pT1b (9)	Not mentioned	RH (8) SH (1)	Not mentioned	Not mentioned	Distant node (7) Peritoneum (3) Lung (2) Liver (1) Skin (1)	DOD (1) AWD (1) NED (7)
Kaplan et al. 1998 [7]	pT1b (1)	39	SH	Chemotherapy and radiotherapy	Positive	Peritoneum	DOD
Batistatou et al. 2000 [8]	pT2a[b] (1)	63	RH	Chemotherapy and radiotherapy	Positive	Lung, mediastinum and loco-regional	DOD
Present study	pT1b (9)	30–68 (50)	RH (9)	Chemotherapy (1) Radiotherapy (1)	Positive (2) Negative (7)	Lung (1)	DOD (1) NED (8)
	pT2b (3)	51–74 (57)	RH (3)	Radiotherapy (2)	Positive (3)	Peritoneum (2) PALN (1)	DOD (3)

SACC = Serous adenocarcinoma of the uterine cervix; RH = radical hysterectomy; SH = simple hysterectomy; PALN = para-aortic lymph node; NED = no evidence of disease; DOD = dead of disease; AWD = alive with disease.
[a] This patient is also included in the report by Zhou et al. [3]. [b] This patient has para-aortic lymph node metastasis.

hysterectomy was 38%. Patients with advanced-stage SACC may have more aggressive tumor behavior than those with common-type adenocarcinoma.

All patients whose tumor recurred had extra-pelvic metastasis in the present review of the literature. Of the 5 distant sites of recurrence, the most frequently reported was distant lymph node (8/21, 38%), followed by peritoneal spread (7/21, 33%), and then lung (4/21, 19%). In our institute, among the 123 patients with common-type adenocarcinoma who underwent radical hysterectomy, 27 (22%) suffered tumor recurrence [11]. Of these, the initial failure sites were inside the pelvis in 10 patients (38%), outside in 15 (58%) and both in 1 patient (4%). Of all distant failure sites, the most frequent were distant nodes (48%) and peritoneal spread (48%), followed by lung (8%). The spread pattern of the initial failure site in patients with SACC or common-type adenocarcinoma is therefore similar in that extra-pelvic metastasis is more frequent, in particular, peritoneal spread and distant node metastasis. Based on these findings, checking for extra-pelvic metastasis should be considered a priority issue for improving the survival of patients with SACC.

In patients with advanced-stage SACC, pelvic control alone usually does not lead to a favorable outcome because of the high incidence of distant metastasis. Whole-pelvic irradiation is generally selected in many institutes as a post-operative adjuvant therapy.

The largest single study of 17 cases, published by Zhou et al. in 1998 [3], revealed several key features. They reported that there was a bimodal age distribution, with 1 peak occurring before the age of 40 years and the second peak after the age of 65. In the present study, however, the mean age of all patients at the time of diagnosis was 52.2 years (range 30–74) and there are 9 cases in patients between the ages of 40 years and 65 years. From the literature review, the mean age of patients with common uterine cervical adenocarcinoma was 48.4 (±12.9) [12]. Thus, at 51 years (range 24–78) [11], the mean age of the

patients with SACC is similar to common-type adenocarcinoma.

Zhou et al. [3] reported that the presenting symptoms were abnormal vaginal bleeding or discharge (13 patients), and abnormal cervical cytology (4 patients). In the present study, 11 patients (92%) presented with abnormal genital bleeding and 1 (8%) with abnormal cervical cytology.

In summary, we have reported detailed clinicopathological features of 12 cases of SACC and reviewed the literature. Patients with pT1b disease may have a favorable outcome with radical surgery. In contrast, patients with more advanced-stage disease had a poor prognosis with established therapy, because of extra-pelvic recurrence. We need to seek effective systemic therapy for advanced-stage SACC.

Further study is warranted and is necessary to confirm the clinical behavior of SACC and to determine optimal therapy.

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Prognostic Impact of the History of Breast Cancer and of Hormone Therapy in Uterine Carcinosarcoma

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Objective: Recent studies reveal an association between hormone therapy for breast cancer (BC), such as tamoxifen (TAM) and toremifene (TOR), and uterine carcinosarcoma (UCS). The aim of this study was to investigate the characteristics and prognosis of patients with UCS after BC and hormone therapy.

Methods: Between January 1997 and December 2007, we treated 51 patients with UCS. The medical records of these patients were reviewed, and factors that influenced their survival were retrospectively analyzed using univariate and multivariate analyses.

Results: Ten (19.6%) of the 51 patients had a history of BC; 6 (11.8%) had received hormone therapy with TAM or TOR. The characteristics of the patients with UCS were similar regardless of whether they had a history of BC or hormone therapy. On univariate analysis, age greater than 56 years, elevated serum lactate dehydrogenase levels, residual tumors, FIGO (International Federation of Gynecology and Obstetrics) stage higher than stage IIIa, and non–endometrioid carcinomatous components were identified as prognostic factors. On multivariate analysis, in addition to residual tumors, FIGO stage higher than stage IIIa, and non–endometrioid carcinomatous components, a history of BC (relative risk, 0.14), a history of TAM use (relative risk, 15.9), and a history of TOR use (relative risk, 16.9) were also identified as independently significant prognostic factors.

Conclusions: Our data suggest that a history of BC and hormone therapy for BC is a risk factor for developing UCS without obvious impacts on the characteristics of UCS. Both of these factors had statistically significant impacts on the prognosis of patients with UCS. Further studies are necessary to clarify and validate these associations.

Key Words: Uterine carcinosarcoma, Breast cancer, Tamoxifen, Toremifene

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Tamoxifen (TAM) is a selective estrogen receptor modulator and is widely used as an adjuvant in patients with estrogen or progesterone receptor–positive breast cancer (BC). Toremifene (TOR), which has a similar structure to TAM, is now also used as hormone therapy for BC. Patients who receive long-term hormone therapy are well known to show an increased incidence of endometrial adenocarcinoma. Patients undergoing TAM therapy longer than 5 years are estimated to have an increased risk of endometrial adenocarcinoma by about 3- to 7-fold[1–3]; although its incidence is reported to be lower in patients undergoing TOR therapy.[4] In early studies, the prognosis of patients with endometrial adenocarcinoma related to TAM was thought to be worse than for those

unrelated to TAM.[3] However, later studies show a comparable prognosis between patients with diseases related to and unrelated to TAM[5,6]; however, this issue remains controversial.

Recently, the association between TAM and uterine carcinosarcoma (UCS) was demonstrated by a number of case reports and case-control studies.[3,7–14] Uterine carcinosarcoma is a tumor with carcinomatous and sarcomatous components and used to be classified as uterine sarcoma. Uterine carcinosarcoma is now considered one of the aggressive subtypes of endometrial adenocarcinoma, and its etiological features and symptoms are thought to be similar to those of endometrial adenocarcinoma. There are studies examining the prognosis of patients with UCS related to TAM.[5,11–13] However, there is still no consensus about the prognosis of patients with UCS related to TAM. Moreover, the prognosis of patients with UCS subsequent to BC without a history of hormone therapy is still unknown.

In the present study, we investigated the characteristics of patients with UCS and whether a history of BC and hormone therapy (e.g., TAM or TOR) can alter their prognosis.

TABLE 1. Characteristics of the patients with UCS in relation to their history of BC and of hormone therapy

	With a History of BC				
Factors	**Hormone Therapy**	**No Hormone Therapy**	**Subtotal**	**Without a History of BC**	**Total**
No. patients	6	4	10	41	51
Age, y	54–80	56–68	54–80	36–79	36–80
Mean, y	68.5	63.3	66.4	60.7	61.8
Median, y	71.5	64.5	67	62	63
≤56	1	1	2	13	15
>56	5	3	8	28	36
Menstrual status					
Premenopausal	0	0	0	8	8
Postmenopausal	6	4	10	33	43
Serum LDH					
Within normal limits	5	2	7	31	38
>Upper normal limits	1	2	3	10	13
Surgical procedures					
TAH + BSO	3	2	5	17	22
RAH or TAH + BSO + PLA	3	2	5	24	29
Sarcomatous component					
Homologous	4	3	7	27	34
Heterologous	2	1	3	14	17
Carcinomatous component					
Endometrioid	6	3	9	28	37
Non-endometrioid	0	1	1	13	14
Residual tumor					
None	6	2	8	34	42
Any	0	2	2	7	9
FIGO stage					
I	4	1	5	14	19
II	1	0	1	6	7
III	1	2	3	17	20
IV	0	1	1	4	5
Classified FIGO stage					
I–IIIa	5	2	7	32	39
IIIb–IV	1	2	3	9	12

TAH, total abdominal hysterectomy; RAH, radical abdominal hysterectomy; BSO, bilateral salpingo-oophorectomy; PLA, pelvic lymphadenectomy.

MATERIALS AND METHODS

Patients

Between January 1997 and December 2007, we treated 51 patients with UCS at the National Cancer Center Hospital, Japan. We reviewed the medical and pathological records of these patients. The data on whether the UCS patients had BC and had undergone hormone therapy, such as TAM and TOR, in addition to other possible prognostic factors of UCS were extracted from their records. According to the Japanese ethical guideline for epidemiologic study, this study was approved by the institutional review board of the National Cancer Center.

Our standard surgical treatment for endometrial cancer, including UCS, consists of total abdominal hysterectomy, bilateral salpingo-oophorectomy, and pelvic lymphadenectomy. We performed radical abdominal hysterectomy in patients with apparent cervical involvement or those with a preoperative diagnosis of cervical carcinoma. Patients with biopsy-proven lymph node metastases also underwent para-aortic lymphadenectomy. In patients with no or superficial myometrial invasion on macroscopic examination of the resected uterus, pelvic lymphadenectomy was omitted and only palpation and sampling of swollen nodes were performed. In patients with extrauterine tumor spread, 6 cycles of postoperative chemotherapy were provided. Paclitaxel/carboplatin combination (TC regimen) and cyclophosphamide/doxorubicin/cisplatin combination (CAP regimen) chemotherapies were administered to 10 and 2 patients, respectively. Other treatment regimens of ifosfamide/cisplatin combination or doxorubicin/dacarbazine combination chemotherapy were administered to 1 patient each. All the patients underwent primary surgical treatment, and no neoadjuvant chemotherapy was performed. Before the start of treatment, written informed consent was obtained from all of the patients.

Statistical Analysis

Patient survival was measured from the day of starting treatment, that is, the day of surgery. Survival curves were determined by the Kaplan-Meier product limit method. Factors influencing survival were analyzed using the log-rank test (univariate) and Cox proportional hazards regression analysis (multivariate). A value of $P < 0.05$ was considered to indicate statistical significance. These analyses were performed using SPSS version 11.0 (SPSS Inc., Chicago, IL). On multivariate analyses, stepwise backward screening was performed with an exclusion $P = 0.05$ to identify independent prognostic factors. Contingency table analysis was performed using Fisher exact test or χ^2 test for trends. Differences in age were examined by nonpaired Student t test.

RESULTS

Associations Between UCS and a History of BC and of Hormone Therapy

The characteristics of the 51 patients with UCS were classified according to their histories of BC and hormone therapy and are summarized in Table 1. Ten (19.6%) of the 51 patients had a history of BC, and 6 patients (11.8%) had

TABLE 2. Characteristics of the patients with UCS and with a history of BC

Case	Age, y	FIGO Stage	BC Laterality	BC to UCS, y	Treatment	Residual Tumor	Carcinomatous Component	Sarcomatous Component	Hormone Therapy, y	Outcome
1	59	Ib	Right	13	TAH + BSO + PLA	No	Endometrioid	Homo	TAM (2)	DOD at 5 mo
2	68	IVb	Left	Synchronous	TAH + BSO + OMT	Yes	Serous	Hetero	No	DOD at 3 mo
3	63	Ib	Left, right	20, 5	TAH + BSO + PLA	No	Endometrioid	Homo	No	NED at 62 mo
4	71	Ic	Unknown	26	TAH + BSO	No	Endometrioid	Homo	TAM (5)	NED at 48 mo
5	80	Ic	Right	4	TAH + BSO	No	Endometrioid	Hetero	TAM (4)	DOD at 7 mo
6	66	IIIb	Right, left	30, 7	TAH + BSO	Yes	Endometrioid	Homo	No	DOD at 35 mo
7	54	Ib	Right	5	TAH + BSO + PLA	No	Endometrioid	Homo	TAM (2)	NED at 63 mo
8	56	IIIa	Right	11	TAH + BSO + PLA	No	Endometrioid	Homo	No	NED at 61 mo
9	75	IIIc	Left	8	TAH + BSO + PLB	No	Endometrioid	Hetero	TOR (5)	DOD at 19 mo
10	72	IIa	Right	7	TAH + BSO + PLA	No	Endometrioid	Homo	TOR (5)	DOD at 22 mo

TAH, total abdominal hysterectomy; BSO, bilateral salpingo-oophorectomy; PLA, pelvic lymphadenectomy; PLB, pelvic lymph node biopsy; OMT, omentectomy; Homo, homologous; Hetero, heterologous; DOD, died of disease; NED, no evidence of disease.

a history of hormone therapy. The median follow-up period of the patients, excluding those who died, was 59 months (range, 12–85 months). Although the patients with a history of hormone therapy were slightly older than those without a history of BC, the difference was not statistically significant (mean age, 68.5 vs 60.7, $P = 0.087$, nonpaired Student t test). The patients with a history of hormone therapy seemed to have earlier stage UCS than those without a history of BC, but this difference was not significant ($P = 0.090$, χ^2 test for trends). The differences in the distributions of the other prognostic factors among the groups were also not statistically significant (P values not shown).

Characteristics of the Patients With a History of BC

The detailed characteristics of the 10 patients with a history of BC are shown in Table 2. All of the 10 patients underwent surgery for BC, and 6 patients (60%) received additional hormone therapy (4 patients with TAM and 2 patients with TOR). The durations of TAM treatment ranged from 2 to 5 years and that of TOR was 5 years. The mean intervals between BC and incidence of UCS in patients with and without a history of hormone therapy were 10.5 years (range, 4–26 years) and 15.3 years (range, 0–30 years), respectively. The interval between BC and incidence of UCS in the 2 patients with bilateral BC was calculated from the time of initial BC diagnosis. The difference in the interval between BC and incidence of UCS was not statistically significant ($P = 0.490$, nonpaired Student t test). The mean interval between hormone therapy cessation and incidence of UCS was 6.7 years (range, 0–21 years).

Analysis of Prognostic Factors

We performed both univariate and multivariate analyses to screen for potential prognostic factors of UCS. Table 3 lists the factors analyzed and the results. The prognostic factors found to be significant from the univariate analysis were age greater than 56 years, elevated serum lactate dehydrogenase (LDH) levels, presence of residual tumors, FIGO (International Federation of Gynecology and Obstetrics) stage higher than stage IIIa, and carcinomatous components other than endometrioid adenocarcinoma. Regarding multivariate analysis, all 11 factors were included in the Cox proportional hazards model, and stepwise backward analysis was performed. The results from this analysis revealed 6 independently significant prognostic factors (Table 3). The presence of residual tumors, FIGO stage higher than stage IIIa, and carcinomatous components other than endometrioid adenocarcinoma were again identified as significant prognostic factors. In addition, a history of BC, history of TAM use, and history of TOR use were also identified as independently significant prognostic factors.

Survival of Patients With UCS in Relation to Their History of BC and of Hormone Therapy

Figure 1 shows the survival curves of the patients with UCS. The 51 patients were divided into 3 groups based on their history of BC and of hormone therapy: patients with UCS not related to BC (n = 41), patients with UCS subsequent

TABLE 3. Univariate and multivariate analyses to identify significant prognostic factors for patients with UCS

	Univariate Analysis	Multivariate Analysis		
Factors	***P***	**Risk Ratio**	**95% CI**	***P***
Age (>56 [n = 36] vs ≤56 [n = 15]), y	0.038	—	—	NS
Menstrual status (postmenopause [n = 43] vs premenopause [n = 8])	0.651	—	—	NS
Serum LDH level (>upper normal limit [n = 13] vs within normal limits [n = 38])	<0.001	—	—	NS
Surgical procedures (RAH or TAH + BSO + PLA [n = 29] vs TAH + BSO [n = 22])	0.153	—	—	NS
Residual tumor (any [n = 9] vs none [n = 42])	<0.001	8.942	2.791–28.647	<0.001
FIGO stage (IIIb–IV [n = 12] vs I–IIIa [n = 39])	<0.001	4.116	1.296–13.074	0.016
Sarcomatous component (heterologous [n = 17] vs homologous [n = 34])	0.107	—	—	NS
Carcinomatous component (non-endometrioid [n = 14] vs endometrioid [n = 37])	0.017	2.896	1.088–7.708	0.033
BC (positive [n = 10] vs negative [n = 41])	0.869	0.139	0.022–0.886	0.037
TAM use (positive [n = 4] vs negative [n = 47])	0.932	15.895	1.461–172.913	0.023
TOR (positive [n = 2] vs negative [n = 49])	0.300	16.872	1.676–169.870	0.016

RAH, radical abdominal hysterectomy; TAH, total abdominal hysterectomy; BSO, bilateral salpingo-oophorectomy; PLA, pelvic lymphadenectomy; NS, not significant.

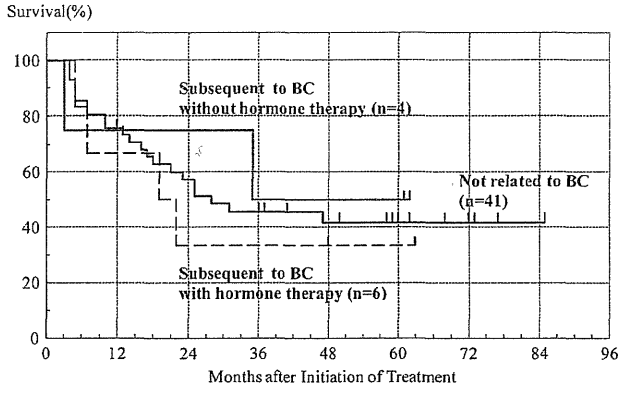

FIGURE 1. Survival curves for patients with UCS with respect to their history of BC and of hormone therapy. The survival rates of patients with UCS unrelated to BC (n = 41), patients with UCS subsequent to BC without hormone therapy (n = 4), and patients with UCS subsequent to BC with hormone therapy (n = 6) are shown in solid black, solid gray, and dotted black lines, respectively.

to BC without hormone therapy (n = 4), and patients with UCS subsequent to BC with hormone therapy (n = 6). Median survival periods and 5-year survival rates of the patients in each of the abovementioned groups were 28 months and 41.8%, 35 months and 50.0%, and 19 months and 33.3%, respectively. The differences in survival rates were not statistically significant on univariate analysis (P = 0.828, log-rank test).

DISCUSSION

In the present study, we investigated the characteristics of patients with UCS and investigated whether a history of BC and/or a history of hormone therapy for BC affected the prognosis of UCS.

We found relatively high incidences of patients with a history of BC (19.6%) and of hormone therapy (11.8%) among those with UCS. A history of pelvic radiation is a well-known risk factor for UCS. Our study population included 2 patients (3.9%) with a history of pelvic radiation (cases 5 and 6; Table 2). Both patients had a history of BC, with one having a history of hormone therapy. Although it is not clear which factors were associated with the development of UCS in these patients, the proportions of patients with histories of BC and hormone therapy were very high. Taking into account the lifetime cumulative incidence of BC that is about 5% in Japanese women,[15] the proportion of surviving female patients with a history of BC and of hormone therapy among the population is probably less than 5%. Thus, our data suggest an etiologic correlation between UCS and hormone therapy consistent with previous reports[3,6,14] and further suggest a similar correlation between UCS and BC itself.

No significant differences in clinical or pathological characteristics were found among UCS patients, without a history of BC, with a history of BC, or with a history of hormone therapy. Kloos et al.[12] suggested that TAM users had more advanced stages of UCS in their series. In contrast, McCluggage et al.[11] and Arenas et al.[13] suggested earlier stages of UCS in their series of TAM users. Our patients with a history of hormone therapy had a relatively early stage of UCS compared with those without BC, although the difference was not statistically significant (P = 0.090, χ^2 test for trends). Meanwhile, the differences in the interval from BC to incidence of UCS (P = 0.490, nonpaired Student t test) and the age of preceding BC (P = 0.353, nonpaired Student t test, data not shown) were not statistically significant between the patients with a history of BC and those with a history of hormone therapy. Taken together, the characteristics of the UCS patients without a history of BC, with a history of BC, and with a history of hormone therapy were not markedly different from each other.

Several prognostic factors of UCS have been reported previously, the most important being the FIGO stage of the tumor.[16,17] The presence of carcinomatous components other than endometrioid adenocarcinoma is also a poor prognostic factor.[18] On the other hand, the prognostic impact of heterologous sarcomatous components is controversial. In the present study, 5 factors were identified as significant prognostic factors using a univariate analysis. Among these, FIGO stage higher than stage IIIa, the presence of residual tumors, and carcinomatous components of non–endometrioid adenocarcinoma remained as independently significant prognostic factors of UCS on a multivariate analysis, whereas age greater than 56 years and elevated serum LDH levels were not independent prognostic factors on the multivariate analysis. We measured serum LDH levels in the routine preoperative systemic evaluations. The serum LDH level is reported to be higher in patients with endometrial cancer compared with healthy controls, but serum LDH level did not correlate with deep myometrial invasion or high histological grade of endometrial cancer.[19] Our data suggest some correlation with prognosis; thus, further assessment of the meaning of elevated serum LDH level is necessary to address its relevance as a predictive measure of UCS. In contrast, the history of TAM or TOR therapy and the history of BC were identified as independently significant prognostic factors on multivariate analysis, but not on univariate analysis. On univariate analysis, even when we analyzed the prognosis by dividing patients into 3 groups, that is, patients without a history of BC (41 patients), with a history of BC (4 patients), and with a history of hormone therapy (6 patients), no significant differences were found (Fig. 1). However, even when we combined the history of TAM or TOR as the history of hormone therapy, the results of multivariate analysis were almost identical. The relative risk of UCS with a history of hormone therapy and the history of BC were 16.410 (95% confidence interval [CI], 2.044–131.746) and 0.138 (95% CI, 0.022–0.867), respectively. As mentioned above, there were no marked differences in the distributions of other characteristics. Thus, the cumulative nonsignificant differences of other prognostic factors, especially FIGO stage higher than stage IIIa, presence of residual tumors, and carcinomatous component of non–endometrioid adenocarcinoma may conceal the significant prognostic impact of the history of BC, TAM, and TOR

on UCS. In previous reports, a case series suggested poor prognosis of patients with a history of TAM,[11–13] and a case-control study of patients with BC suggested poorer prognosis for patients with a history of long-term TAM use among those with endometrial cancer, including UCS.[3] On the other hand, a follow-up study of patients with BC found no prognostic impact of TAM use in patients with UCS subsequent to BC.[5] To our knowledge, there have been no previous studies analyzing the prognostic impact of the history of BC itself and of hormone therapy among patients with UCS, including those patients without preceding BC. We found that the history of BC was a significantly better prognostic factor of UCS, whereas the history of TAM and TOR treatment was a significantly poor prognostic factor of UCS on our multivariate analysis.

Our data suggest that histories of both BC and hormone therapy for BC are important risk factors for UCS, whereas characteristics of the UCS are similar regardless of the presence of these risk factors. Moreover, the history of BC appears to be a good prognostic factor in UCS patients, whereas a history of hormone therapy is a poor prognostic factor. However, because of the rarity of UCS occurrence and UCS related to BC as shown by Lavie et al,[20] our study is based on a small cohort of patients from a single institution. This is a limitation of our study, along with the retrospective nature of our study. Thus, the results from our study need to be validated by future studies to clarify the association between prognosis of UCS and a history of BC and/or of hormone therapy.

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Indoleamine-2,3-dioxygenase, an immunosuppressive enzyme that inhibits natural killer cell function, as a useful target for ovarian cancer therapy

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Abstract. This study examined the role of the immunosuppressive enzyme indoleamine-2,3-dioxygenase (IDO) in ovarian cancer progression, and the possible application of this enzyme as a target for ovarian cancer therapy. We transfected a short hairpin RNA vector targeting IDO into the human ovarian cancer cell line SKOV-3, that constitutively expresses IDO and established an IDO downregulated cell line (SKOV-3/shIDO) to determine whether inhibition of IDO mediates the progression of ovarian cancer. IDO downregulation suppressed tumor growth and peritoneal dissemination *in vivo*, without influencing cancer cell growth. Moreover, IDO downregulation enhanced the sensitivity of cancer cells to natural killer (NK) cells *in vitro*, and promoted NK cell accumulation in the tumor stroma *in vivo*. These findings indicate that downregulation of IDO controls ovarian cancer progression by activating NK cells, suggesting IDO targeting as a potential therapy for ovarian cancer.

Introduction

Ovarian cancer is the fifth leading cause of cancer-related-death in the US. Approximately 22,000 women suffered from ovarian cancer in 2010, about 14,000 of whom died of this disease (1). Since most patients with early-stage ovarian cancer seldom have any symptoms, at the time of diagnosis, over 75% are already in advanced stages with peritoneal dissemination and ascites, which are the typical symptoms (2). The standard treatment for ovarian cancer is cytoreductive surgery with platinum/taxane combination chemotherapy. Ovarian cancer is mostly sensitive to chemotherapy (3,4), but becomes ineffective over time due to the development of chemoresistance. The 5-year survival rate is only 40%, and has not improved in the last decade (1). Therefore, new strategies, such as immunotherapy and molecular-targeted therapy, may prove useful in improving the prognosis of ovarian cancer. The most common form of ovarian cancer spread is peritoneal dissemination (2). Although the mechanism involved in this process are largely unknown, studies indicate that, immunotolerance induction plays an important role (5,6).

Indoleamine-2,3-dioxygenase (IDO) is an enzyme that catalyzes the first and rate-limiting step in the kynurenine pathway of tryptophan catabolism. IDO was originally discovered in 1967 (7,8) in the rabbit small intestine and was first purified in 1978 (9). Subsequently, it was reported that IDO could be induced in the mouse lung with either influenza virus infection (10) or bacterial endotoxin shock (11). Proinflammatory mediators, such as interferon-γ or other cytokines can also stimulate IDO induction (12). The first study that described IDO as an immunosuppressant found that IDO in the mouse placenta prevented rejection of the allogeneic fetus (13). Recently, it was clarified that IDO can induce immunotolerance in patients with autoimmune diseases (14-17), chronic infections (18), and cancer (19). It was also reported that most human tumors express IDO (19) and that IDO can contribute to tumor-induced immunosuppression by starving T cells, which are sensitive to tryptophan deficiency. In this situation, tumor cells can escape immune surveillance via the action of IDO (13). Natural killer (NK) cells are important members of the innate immune system, which plays a role in inhibiting the growth and dissemination of several kinds of tumors (20). A series of receptors expressed by NK cells are known to modulate the cytotoxicity of NK cells against tumors (21). The tryptophan-derived catabolic kynurenine can reduce

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NK cell number and weaken NK cell cytotoxicity by inhibiting NK cell receptors, thus contributing to tumor progression (22). IDO is frequently expressed in many cancers such as gastric, pancreatic, colorectal, and prostate cancers (19). In the gynecological field, IDO expression has been observed in cervical, endometrial, and ovarian cancer (19), and associations between its expression and the prognosis of these cancers have been reported (23-26).

RNA interference (RNAi) is a good technique for gene silencing, that involves a post-transcriptional gene-silencing mechanism (27). Among the different types of RNAi techniques, the use of small interfering RNAs (siRNAs) effectively suppresses gene expression, but the suppression is transient (28), which limits its therapeutic use. Short hairpin RNAs (shRNAs) driven by polymerase III promoters have been developed as an alternative strategy to attain long-term stable target gene silencing and understand the consequence of stable silencing (29,30).

In this study, we used an shRNA vector targeting IDO to silence IDO expression in an IDO-expressing ovarian cancer cell line to clarify the relationship between IDO expression and peritoneal dissemination of ovarian cancer. Moreover, we investigated the function of NK cells in ovarian cancer progression in order to develop an IDO-targeted molecular therapy that inhibits peritoneal dissemination.

Materials and methods

Cell culture. The human ovarian cancer cell line SKOV-3 (31) (American Type Culture Collection, Manassas, VA) were cultured in RPMI-1640 medium (Gibco, Grand Island, NY) containing 10% inactivated fetal calf serum (Sigma, St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37°C in a 5% CO_2 atmosphere for no longer than 8 weeks after recovery from frozen stocks.

The NK cell line KHYG-1 (32) was purchased from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). Cells were cultured in RPMI-1640 medium supplemented with 100 nM of human interleukin-2 (R&D Systems, Minneapolis, MN) and 10% inactivated fetal calf serum (Sigma), at 37°C in a 5% CO_2 atmosphere for no longer than 8 weeks after recovery from frozen stocks.

Antibodies. Anti-human IDO monoclonal antibody was prepared as previously reported (33). Anti-human actin antibody (Sigma) and anti-mouse CD49b antibody (R&D) were used according to the manufacturer's protocols.

shRNA stable cell line and control cell line. The DNA oligonucleotides encoding shRNA targeting the IDO gene (forward: 5'-CACCGGGGCAGATTATAAGAATTACGTGTGCTGTCC GTAATTCTTGTAGTCTGCTCCTTTTT-3', reverse: 5'-CCC CGTCTAATATTCTTAATGCACACGACAGGCATTAAGA ACATCAGACGAGGAAAAATACG-3') were synthesized, annealed, and inserted into the *Bsp*MI site of the vector piGENE PURhU6 (34), which contained a human U6 promoter, and a puromycin resistance gene. The shRNA expression plasmid (piGENE PURhU6/shIDO) and control plasmid (piGENE PURhU6) were transfected into SKOV-3 using Lipofectamine LTX and Plus Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cells were selected using 0.5 μg/ml puromycin (Calbiochem, Darmstadt, Germany). Resistant clones were obtained after 4 weeks as SKOV-3/shIDO, SKOV-3/Mock. The cells were subsequently maintained in the presence of 0.5 μg/ml puromycin.

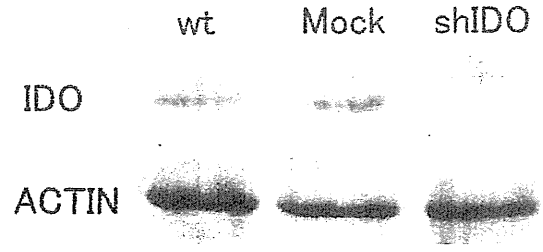

Figure 1. Western blot of parental cells (wt) and control vector-transfected cells (Mock) showing evident IDO expression. In contrast, the shIDO vector-transfected cells (shIDO) did not show IDO expression.

Western blotting. Protein (10 μg) extracted from a homogenate of cultured cells was mixed with 2X SDS-PAGE sample buffer [120 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.004% bromophenol blue, and 10% 2-mercaptoethanol]. The mixture was heated at 95°C for 2 min, and electrophoresed on a 0.1% SDS-10% polyacrylamide gel, and then the proteins were blotted onto a polyfluorovinylidene membrane. The menbranes were blocked with Non-Protein Blocking Agent (ATTO Corporation, Tokyo, Japan) at room temperature for 1 h, and incubated with anti-human IDO monoclonal antibody (1:1,000) and anti-human actin polyclonal antibody (1:200) for 1 h at room temperature. The membrane was washed with phosphate-buffered saline (PBS)-Tween-20 three times, and then incubated with horseradish peroxidase-conjugated secondary anti-mouse antibody (Thermo, Rockford, IL) or anti-rabbit antibody (Thermo). Signals were detected by chemiluminescence (ECL kit; Amersham Biosciences, Piscataway, NJ) on X-ray film.

In vitro cell growth kinetics. SKOV-3/shIDO and SKOV-3/Mock cells (500 of each line) were seeded into a 96-well plate, and cultured in RPMI-1640 medium containing 10% fetal calf serum. Every 24 h, cells were counted using a colorimetric assay with the Cell Proliferation kit II (XTT) (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany), and a growth curve was drawn from the results.

Sensitivity of transfectants to NK cells in vitro. The sensitivity of SKOV-3/shIDO and SKOV-3/Mock cells to NK cells was investigated by colorimetric assay using XTT. SKOV-3/shIDO and SKOV-3/Mock cells (500 of each line) were seeded into a 96-well plate and co-cultured with KHYG-1 cells (0, 500, 1000, or 2000 cells) in RPMI-1640 medium containing 10% fetal calf serum for 72 h. After 3 washes with PBS to exclude KHYG-1 cells completely, the viable cell count was determined by colorimetric assay and calculated as the percent of control cells (cultured without KHYG-1 cells).

Experimental animals. Four- to six-week-old female BALB/c nude mice (Japan Clea Laboratories, Tokyo, Japan) were used.

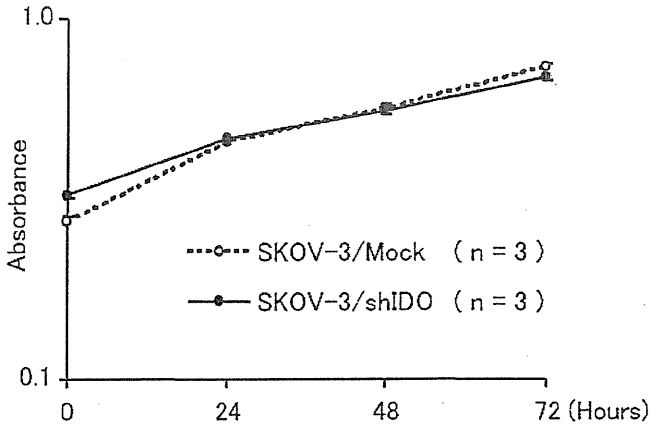

Figure 2. Cell growth curves of SKOV-3/shIDO and SKOV-3/Mock (control) cells. There was no significant difference between the 2 groups. Results are expressed as mean ± SD.

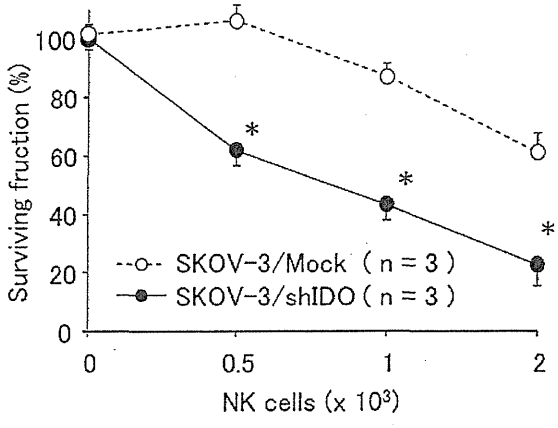

Figure 3. The percent of viable tumor cells co-cultured with NK cells. The percent survival of SKOV-3/shIDO cells was significantly lower than that of control cells. *P<0.01. The results are expressed as mean ± SD.

All animal experiments were conducted according to the institutional and national guidelines for animal experiments.

Subcutaneous tumor growth in vivo. SKOV-3/shIDO and SKOV-3/Mock cells ($5x10^6$ cells of each line) were inoculated subcutaneously into the back of mice to induce tumor growth. The tumor volume [(long diameter) x (short diameter)^2 x 1/2] was measured twice a week to draw a tumor growth curve.

Peritoneal dissemination in vivo. SKOV-3/shIDO and SKOV-3/Mock cells ($5x10^6$ cells of each line) were injected intraperitoneally into nude mice, and the mice were observed until death. A survival curve was constructed using the Kaplan-Meier method. The mice were checked for survival twice a day.

Immunohistochemical staining. At one week after subcutaneous tumor cell inoculation, mice were sacrificed under isoflurane anesthesia, and the tumor was removed. After formalin fixation, paraffin sections were prepared, deparaffinized, and treated with hydrogen peroxide for 30 min to block endogenous peroxidase. The sections were then reacted with a 1:10 dilution (5 μg/ml) of anti-mouse CD49b primary antibody for 16 h at

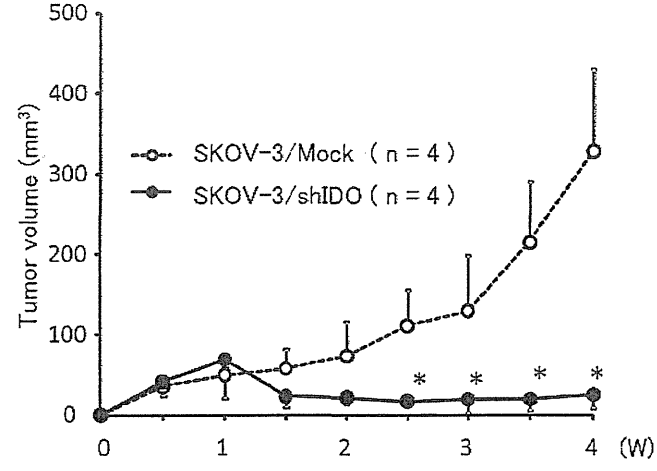

Figure 4. Subcutaneous tumor growth curves of SKOV-3/shIDO and control cells. Both groups of cells formed small nodules one week after inoculation. Subsequently, the tumors in the control group enlarged, whereas those in the SKOV-3/shIDO group disappeared. *P<0.05. Mean ± SD.

room temperature, washed 3 times washes with PBS, and then incubated with enzyme-conjugated streptavidin for 30 min. The sections were again washed with PBS 3 times, and color was developed using the diaminobenzidin method. The number of stained NK cells was counted under high-power magnification (x400).

Statistical analysis. Except for the comparison of survival curves, the test of significance between the 2 groups was performed using Student's t-test. The generalized Wilcoxon test was used to compare survival curves between the 2 groups. A P-value of <0.05 was considered significant.

Results

Establishing an IDO-downregulated cell line. Fig. 1 shows the results of Western blot analysis of the shIDO expression vector- or control vector-transfected ovarian cancer cell line SKOV-3. Parental cells (wt) and control vector-transfected cells (Mock) showed evident IDO expression. In contrast, the shIDO expression vector-transfected cells (shIDO) did not show IDO expression, confirming IDO downregulation in the SKOV-3/shIDO cell line.

In vitro cell growth kinetics. Growth curve analyses of SKOV-3/shIDO and SKOV-3/Mock cells showed no significant difference between the two groups, suggestiong that the downregulation of IDO did not affect cell growth *in vitro* (Fig. 2).

Sensitivity of transfectants against NK cells in vitro. The proportion of viable tumor cells co-cultured with NK cells is shown in Fig. 3. The percent survival of SKOV-3/shIDO cells was significantly lower than that of the control cells, indicating that the downregulation of IDO reinforced the sensitivity of tumor cells against NK cells.

Tumor growth in vivo. Both SKOV-3/shIDO and control cells formed small nodules one week after inoculation (Fig. 4). Subsequently, the tumors in the control group were enlarged,

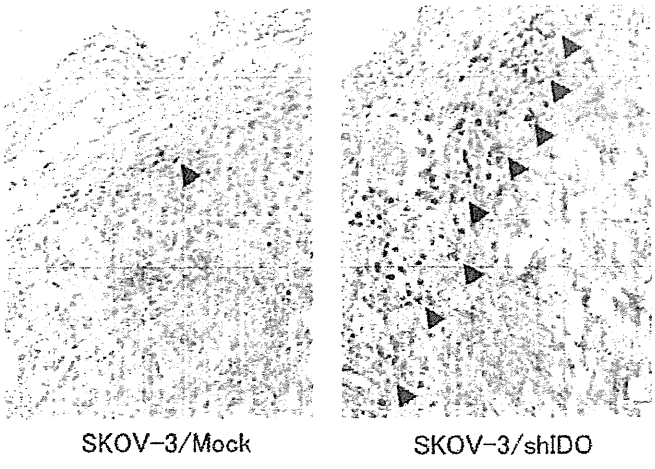

Figure 5. CD49b expression in SKOV-3/shIDO and control subcutaneous tumors. The black arrowheads indicate NK cells accumulating in the tumor stroma.

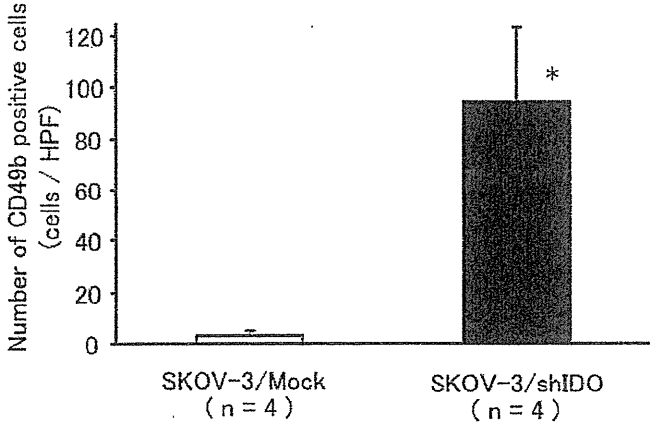

Figure 6. The number of NK cells per high-power field. The number of NK cells (94±29) that accumulated in the SKOV-3/shIDO tumors was significantly higher than that (3±2) in the control tumors. *P<0.01. Mean ± SD.

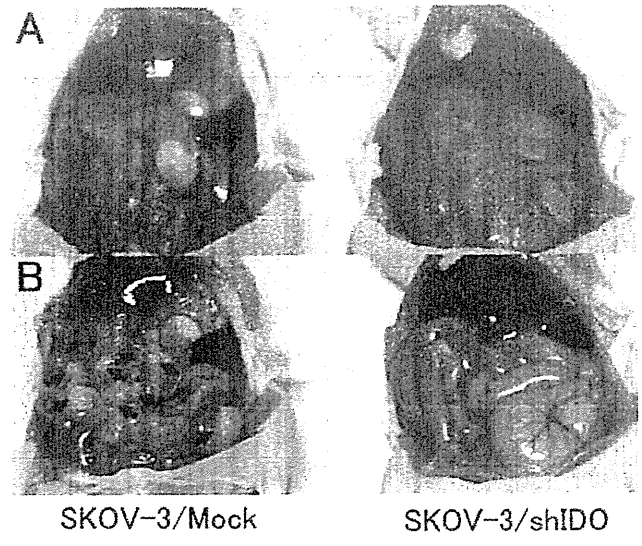

Figure 7. Peritoneal dissemination and ascites accumulation at 4 weeks after the intraperitoneal inoculation of SKOV-3/shIDO or control cells. Ascites accumulation (A). Peritoneal dissemination (B). The black arrow indicates disseminated peritoneal tumors.

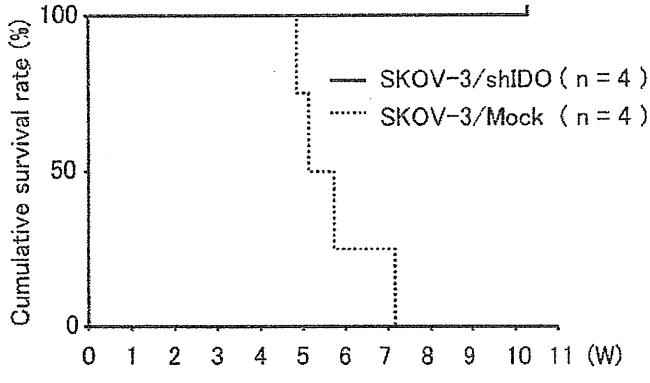

Figure 8. Survival curves of intraperitoneally inoculated mice. All control cell-inoculated mice died of peritoneal dissemination with ascites within 50 days after inoculation, whereas all SKOV-3/shIDO cell-inoculated mice survived for longer than 70 days. *P<0.01.

whereas those in the SKOV-3/shIDO group were reduced, suggesting that the downregulation of IDO inhibited tumor growth *in vivo*.

Number of NK cells in the tumor stroma. Immunostaining of NK cells (black arrowhead) shows accumulation of NK cells in the stroma of SKOV-3/shIDO and control subcutaneous tumors (Fig. 5). The number of NK cells (94±29) that accumulated in the SKOV-3/shIDO tumors was significantly higher than that (3±2) in the control tumors (P<0.01) (Fig. 6). These results suggest that the downregulation of IDO promoted NK cell accumulation around the tumor.

Peritoneal dissemination in vivo. Four weeks after intraperitoneal tumor cell inoculation, mice with intraperitoneally-injected control cells demonstrated bloody ascites and marked peritoneal dissemination, whereas those receiving the intraperitoneal injection of SKOV-3/shIDO cells showed no abnormal changes (Fig. 7A and B). All control cell-inoculated mice died of peritoneal dissemination with ascites within 50 days after inoculation, whereas all SKOV-3/shIDO cell-inoculated mice survived longer than 70 days after inoculation (P<0.01) (Fig. 8). Thus, downregulating IDO inhibited peritoneal dissemination formation and ascites accumulation in tumor-inoculated mice.

Discussion

The experiments described herein aimed to clarify the relationship between the immunosuppressive enzyme IDO and ovarian cancer progression, as well as to develop a molecular therapy-targeting IDO. Previously, we transfected an IDO expression vector into a non-IDO-expressing human ovarian cancer cell line and established an IDO-expressing cell line to examine the relationship between IDO expression and ovarian cancer progression, especially in term of peritoneal dissemination *in vivo* (35). In the present study, we utilized an shRNA expression vector targeting the IDO gene to examine whether inhibition of IDO can control peritoneal dissemination

of ovarian cancer. We found that the downregulation of IDO expression did not influence cancer cell growth *in vitro*, but controlled tumor growth and peritoneal dissemination *in vivo*. In addition, the downregulation of IDO reinforced the sensitivity of cancer cells to NK cells *in vitro* and promoted NK cell accumulation in the tumor stroma *in vivo*. These findings indicate that the downregulation of IDO controls peritoneal dissemination of ovarian cancer by promoting NK cell accumulation in tumors, suggesting that IDO is a useful therapeutic target for patients with ovarian cancer.

Lack of the essential amino acid tryptophan and accumulation of its metabolite, kynurenine, inhibit cell growth and induce apoptosis. T cells are particularly sensitive to this type of stress (13). Regarding the mechanism of cancer cell immunotolerance, IDO has been shown to promote local tryptophan depletion, resulting in T-cell function suppression around IDO-expressing cancer cells and local immunotolerance (19). The possibility cannot be excluded that IDO expression is involved in the immunotolerance of ovarian cancer through such a T cell-mediated mechanism. We initially obtained a murine ovarian tumor cell line (OV2944-HM-1) with the ability to develop into subcutaneous tumor and disseminate peritoneally in immunocompetent mice. However, IDO was hardly detected in this cell line, according to the results of Western blot analysis using an anti-mouse IDO antibody (data not shown). Therefore, we chose the human ovarian cancer cell line (SKOV-3) that constitutively expresses IDO and implanted them in nude mice. Nude mice congenitally lack T cells; therefore, in this experimental system, we could not examine the effect of IDO on T-cell function.

It has been reported that IDO induces the accumulation of the tryptophan metabolite kynurenine, which suppresses NK cell receptor expression, and thereby inhibits NK cell function (22). Similarly, in our previous experiments, IDO expression inhibited the cytotoxic activity of NK cells *in vitro* and suppressed NK cell accumulation in the tumor stroma *in vivo* (35). Herein, we demonstrated that IDO downregulation enhanced the sensitivity of cancer cells to NK cells *in vitro* and promoted NK cell accumulation in the tumor stroma *in vivo*. Thus, the downregulation of IDO reinforced the sensitivity of cancer cells to NK cells, mediating peritoneal dissemination and growth of ovarian cancer.

Typical methods of inhibiting IDO function include the use of 1-methly-tryptophan (1-MT) and gene silencing by RNAi. In IDO-catalyzed tryptophan metabolism, 1-MT competes with tryptophan for IDO, acting as an IDO inhibitor (36). Inaba *et al* reported that the oral administration of 1-MT to the host suppressed the tumor growth of IDO-overexpressing ovarian cancer cells with enhanced proliferative activity (26). Similarly, in our previous study, we showed that oral administration of 1-MT inhibited the tumor growth potential of IDO-transfected ovarian cancer cells with enhanced proliferative activity (35). In our study, mice given 1-MT orally showed no fatal side effects (35). These findings suggest the possibility of IDO-targeted molecular therapy for ovarian cancer using the oral administration of 1-MT or its analogues. Muller *et al* reported that the combination of 1-MT with paclitaxel synergistically regressed an autochthonous breast cancer (37). In addition, Inaba *et al* demonstrated that treatment with 1-MT plus paclitaxel synergistically prolonged mouse survival compared to treatment with paclitaxel alone in an IDO-overexpressing ovarian cancer peritoneal carcinomatosis model (26). Since paclitaxel is a key drug in the chemotherapy of ovarian cancer, the combined use of such an anticancer drug and targeted therapy against IDO may be advantageous in treating ovarian cancer.

Compared to 1-MT treatment, RNAi demonstrates higher potency and efficiency (38). To date, chemically synthesized siRNA and vector-mediated expression of shRNA are the more commonly used RNAi techniques for gene silencing in mammalian cells (30,39). Although siRNA can be more easily transfected into cancer cells, and its silencing function is more effective, its function is transient. The remarkable advantages of shRNA is that the inhibition of target genes can last for weeks or even months, making it possible to elucidate the consequences of long-term stable silencing of a gene (30). In actual clinical settings, nanoparticle-based vectors (40) or viral-based expression vectors could be used to deliver the IDO shRNA to the cancer cells.

The results of this study demonstrate that the downregulation of IDO in human ovarian cancer cells constitutively expressing IDO inhibits ovarian cancer progression, suggesting that the use of IDO-targeted shRNA as a potentially effective molecular-targeted therapy for ovarian cancer.

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Cetuximab inhibits the growth of mucinous ovarian carcinoma tumor cells lacking *KRAS* gene mutations

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Abstract. The purpose of this study was to explore the possibility of targeted molecular therapy with anti-epidermal growth factor receptor (anti-EGFR) antibody (cetuximab) for the treatment of mucinous ovarian carcinoma. We analyzed EGFR protein expression and *KRAS* gene mutations in 5 mucinous ovarian carcinoma cell lines RMUG-L, RMUG-S, MN-1, OMC-1 and MCAS and evaluated the *in vitro* and *in vivo* effects of cetuximab on each. EGFR expression was observed in all cell lines except for MN-1 cells, and a *KRAS* gene mutation at codon 12 was detected only in the MCAS cell line. Cetuximab inhibited RMUG-L and OMC-1 cell growth *in vitro* and completely blocked RMUG-L tumor growth *in vivo*. On the other hand, cetuximab did not affect MCAS cell growth *in vitro* and only partially reduced the MCAS tumor growth *in vivo*. These results suggest the possibility of targeted molecular therapy with cetuximab for mucinous ovarian carcinoma cells lacking a *KRAS* gene mutation.

Introduction

Ovarian cancer is the fifth leading cause of cancer-related-death in the United States. Ovarian cancer was reported in ~22,000 women in 2010, ~14,000 of whom ultimately died of this disease (1). Since most patients with early-stage ovarian cancer seldom have symptoms, by the time they are diagnosed, >75% are already in the advanced stage (2). The standard treatment for ovarian cancer is cytoreductive surgery with platinum/taxane combination chemotherapy. Although ovarian cancer is generally sensitive to chemotherapy (3,4), there are cases that exhibit both natively drug-resistant tumors as well as tumors that eventually acquire drug tolerance. The 5-year survival rate is only 40% and has not improved in the last decade (1). Therefore, new strategies, especially targeted molecular therapy, require more attention in order to improve the prognosis of ovarian cancer.

Mucinous ovarian adenocarcinoma (MAC) accounts for 10-14% of all types of epithelial ovarian cancers (EOC) (5,6). Compared to serous adenocarcinoma (SAC), which is the most common histopathologic subgroup of EOC, MAC is relatively resistant to the conventional platinum or taxane-based chemotherapy, thereby leading to a poor prognosis (7-10). It has been reported that MAC differs from SAC pathologically and cytogenetically, and more closely resembles colorectal cancer (11). These results suggest that therapeutic agents that are effective in treating colorectal cancer may also be effective for treating MAC.

Epidermal growth factor (EGF) and its receptor (EGFR) are reportedly involved in the growth and extension of malignant tumors (12). In particular, EGFR overexpression has been observed in various malignant tumors (13). Further, EGFR overexpression has been reported to be a poor prognostic factor for various malignant tumors (14,15). It has been reported that EGFR is expressed in 48% of MAC tumors, and its expression is correlated with the histologic grade, stage and death rate (16).

Cetuximab, an anti-EGFR monoclonal antibody, is a molecular-targeted therapeutic agent that was produced as a human-mouse chimeric antibody; it has a higher binding affinity for EGFR than natural ligands and inhibits tyrosine kinase phosphorylation (17,18). In addition, cetuximab reportedly induces EGFR internalization and degradation (19). Recently, it has been widely used in the medical treatment of colorectal cancer (20).

The purpose of this study was to explore the possibility of molecular-targeted therapy using anti-EGFR antibody (cetuximab) for MAC as a potential new treatment for this disease.

Materials and methods

Cell culture. The 5 MAC cell lines used in this study (RMUG-L, RMUG-S, MN-1, OMC-1 and MCAS) (21-25) were obtained as follows: the RMUG-L and RMUG-S lines were obtained from Dr Daisuke Aoki (Keio University, Tokyo, Japan); the MN-1

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Key words: ovarian cancer, mucinous ovarian carcinoma, cetuximab, epidermal growth factor receptor, KRAS

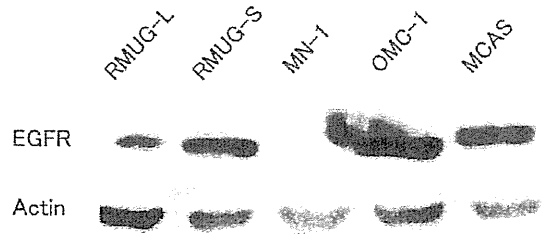

Figure 1. Western blotting using an anti-EGFR polyclonal antibody. EGFR expression was detected at the position corresponding to a molecular weight of 170 kDa in all cell lines except for MN-1 cells.

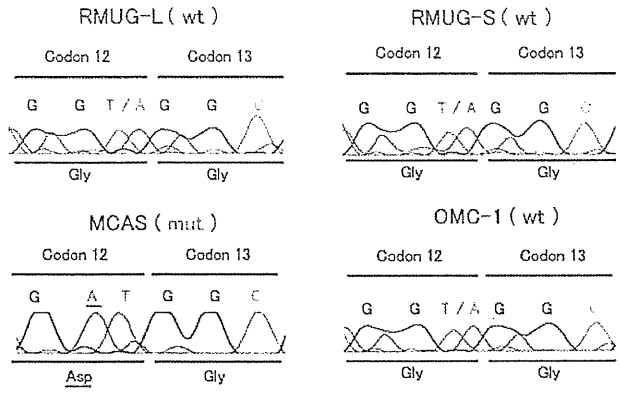

Figure 2. DNA sequence analysis of the *KRAS* gene at codon 12 in exon 2 in 4 MAC cell lines. No mutations were detected in RMUG-L, RMUG-S and OMC-1 cell lines. A point mutation [GGT (Gly) to GAT (Asp)] was observed only in the MCAS cell line.

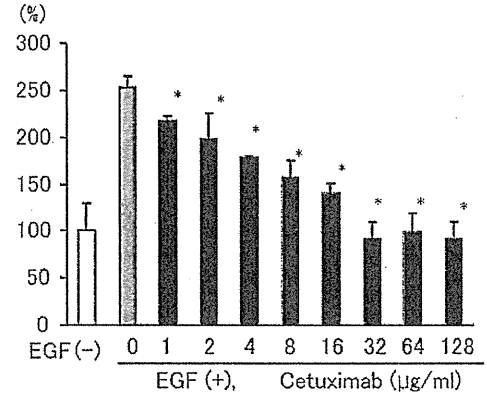

Figure 3. Analysis of the inhibitory effect of cetuximab on *in vitro* cell growth of RMUG-L cells. Cetuximab inhibited the *in vitro* cell growth in a concentration-dependent manner. *P<0.01. Results are expressed as mean ± SD.

line, from Dr Yasuhiko Kiyozuka (Kansai Medical University, Osaka, Japan); the OMC-1 line, from Dr Tsuyoshi Saito (School of Medicine, Sapporo Medical University, Sapporo, Japan); and the MCAS cell line, purchased from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). These cell lines were maintained in D-MEM/Ham's F-12 medium (DMEM/F12, Gibco, Grand Island, NY) containing 10% inactivated fetal calf serum (Sigma, St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37°C in a 5% CO_2 atmosphere for no longer than 8 weeks after recovery from frozen stocks.

Antibodies. Both the anti-EGFR polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and the anti-human actin polyclonal antibody (Sigma) were used according to the manufacturer's protocols.

Anti-EGFR monoclonal antibody (cetuximab). Cetuximab was purchased from Bristol-Myers Squibb (Tokyo, Japan) and used undiluted at a concentration of 2 mg/ml in animal experiments.

Western blotting. Protein extracted (10 μg) from a homogenate of cultured cells was mixed with 2X SDS-PAGE sample buffer [120 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.004% bromophenol blue, and 10% 2-mercaptoethanol]. The mixture was heated at 95°C for 2 min, electrophoresed on a 0.1-5% SDS-polyacrylamide gel, and then the proteins were blotted onto a polyfluorovinylidene membrane. The membranes were blocked with Non-Protein Blocking Agent (ATTO Corporation, Tokyo, Japan) at room temperature for 1 h, and incubated with anti-EGFR polyclonal antibody (1:1,000) and anti-human actin polyclonal antibody (1:200) for 1 h at room temperature. Each membrane was washed with phosphate-buffered saline (PBS)-Tween-20 three times, and then incubated with a horseradish peroxidase-conjugated secondary anti-rabbit antibody (Thermo, Rockford, IL). Signals were detected by chemiluminescence (ECL kit, Amersham Biosciences, Piscataway, NJ) on X-ray film.

KRAS gene mutations. Each of the 5 MAC cell lines were analyzed for KRAS gene mutations. Genomic DNA was extracted from cells by using a QIAamp DNA Mini kit (Qiagen, Valencia, CA). The hot-spots (exon 2) of KRAS gene mutations were amplified by PCR with EX Taq (Takara, Tokyo, Japan) and primers as described previously (26) and sequenced to confirm the presence or absence of mutations by using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Applied Biosystems Division, Darmstadt, Germany) with the ABI PRISM 310 genetic analyzer (Perkin Elmer).

Effects of cetuximab in vitro. RMUG-L, OMC-1 and MCAS were cultured in DMEM/F12 medium supplemented with 100 pg/ml EGF (R&D Systems, Minneapolis, MN) without fetal calf serum and exposed to cetuximab at concentrations of 0-128 μg/ml. To examine the inhibitory effect of cetuximab on cell growth, 5,000 cells/well were dispensed into 96-well plates. After 48 h, the viable cell count was determined by a colorimetric assay with the Cell Proliferation kit II (XTT) (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) and calculated as the percent of control cells (cultured without cetuximab).

Experimental animals. Four- to six-week-old female BALB/c nude mice (Japan Clea Laboratories, Tokyo, Japan) were used. All animal experiments were conducted according to the institutional and national guidelines for animal experiments.